JRC Scientific and Technical Reports

The Use of Chemical Reactivity Assays in Toxicity Prediction

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EUR 24870 EN - 2011

JRC EUROPEAN COMMISSION

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JRC65567

EUR 24870 EN
ISBN 978-92-79-20641-2
ISSN **1831-9424**
doi:10.2788/32962

Luxembourg: Publications Office of the European Union

Printed in Italy

ABSTRACT

The use of so-called "*in chemico*" methodology - abiotic assays that measure chemical reactivity - is gaining ground as relevant and reliable means of toxicity prediction. In this report we explain the basis of the *in chemico* approach to toxicity prediction and we review the studies that have developed the concept and its practical application since the 1930s, with special attention being paid to studies aimed at the development of Quantitative Structure-Activity Relationship (QSAR) models and read-across approaches. The studies covered in this review are limited to non-enzymatic experiments and to nucleophiles up to 50 amino acids. The main applications identified are related to the assessment of skin sensitisation, aquatic toxicity and hepatotoxicity. Various experimental measures of nucleophile depletion or adduct formation have been proposed as chemical reactivity descriptors, but no single protocol has emerged as the most generally useful. It is concluded that *in chemico* approaches provide a promising means of toxicity prediction within their applicability domains and should be further developed and investigated as alternative methods to animal testing, especially when used in the context of integrated testing strategies based on the use of multiple non-animal methods.

LIST OF ABBREVIATIONS

ACD	Allergic Contact Dermatitis
AEI	Activation Energy Index
DMF	Dimethyl fumarate
DNA	Deoxyribonucleic acid
ECHA	European Chemicals Agency
EC3	Effect Concentration 3 (concentration of test chemical that causes a SI of 3)
EC_{50}	Half maximum effective concentration (concentration of test chemical which induces a response half way between the base line and maximum after a certain time of exposure)
E_{HOMO}	Energy of the HOMO orbital
E_{LUMO}	Energy of the LUMO orbital
EU	European Union
GPMT	Guinea-Pig Maximization Test
GSH	Glutathione
HHPA	Hexahydrophthalic anhydride
HOMO	Highest Occupied Molecular Orbital
HPLC	High-Performance Liquid Chromatography
IGC_{50}	Inhibited Growth cells by 50% (concentration of chemical inhibits cell growth by 50%)
JRC	Joint Research Centre
LC/MS	Liquid Chromatography / Mass Spectrometry
LD_{50}	Median lethal dose (concentration of chemical necessary to kill half of the members of the tested population)
LLNA	Local Lymph Node Assay
LUMO	Lowest Unoccupied Molecular Orbital
MCI	5-Chloro-2-methylisothiazol-3-one
MEST	Mouse Ear Swelling Test
MHF	Methylhydrogen fumarate
MI	2-Methylisothiazol-3-one
NBT	4-nitrobenzenethiol
NBP	4-nitrobenzylpyridine
NMR	Nuclear Magnetic Resonance
OECD	Organisation for Economic Cooperation and Development
P	Lipophilicity
PPD	p-phenylendiamine
QMM	Quantitative Mechanistic Modelling
QSAR	Quantitative Structure-Activity Relationship
QSPR	Quantitative Structure-Property Relationship
RAI	Relative Alkylation Index
RC_{50}	Reactive Concentration 50% (concentration of test chemical that reacts with 50% of the model chemical)
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SAR	Structure-Activity Relationship
SI	Stimulation Index
S_NAr	Aromatic Nucleophilic Substitution reaction
S_N1	Nucleophilic Substitution Type 1
S_N2	Nucleophilic Substitution Type 2
SPR	Surface Plasmon Resonance
UV	Ultra-violet

CONTENTS

1. Introduction

A range of toxicological effects of chemicals can be the result of a molecular initiating event in which the xenobiotic molecule reacts covalently with a macromolecule. For example, covalent binding to DNA may lead to mutagenesis and genotoxicity, whereas binding to an immunoprotein may lead to (skin or respiratory) sensitisation. In such reactions, the molecule is typically acting as an electrophile that reacts with nucleophilic centres in one or more macromolecules (typically DNA or protein). Electrophilic centres in electrophiles typically include carbonyl groups, halogenated carbons and unsaturated bonds. Nucleophilic centres typically include sulphur, nitrogen and oxygen atoms. In addition, certain xenobiotics may be reactive by acting as nucleophiles or as reactive oxygen species.

The principle of relating organic reaction chemistry to toxicology has led to the development of a wide range of approaches that use experimental or calculated measures of reactivity as the basis for predicting chemical toxicity. So-called "*in chemico*" methodology refers to a variety of abiotic assays that measure chemical reactivity. Although the term "*in chemico*" has gained ground (Aptula & Roberts, 2006) in the last five years, chemical reactivity models have been reported since the 1930s (Landsteiner & Jacobs, 1936).

The use of *in chemico* methods for toxicity prediction is based on the assumption that chemical reactivity and toxicity are proportionally related, *i.e.* the most reactive chemical will be the most toxic. The absolute value of chemical reactivity is not intrinsically meaningful in toxicological terms, since the reactivity-toxicity relationship is not completely linear, with many factors other than chemical reactivity contributing to toxicity. Nevertheless, within a group of mechanistically related chemicals, a quantitative measure of chemical reactivity can be used to scale the relative toxic potencies of group members. In some studies, information on chemical reactivity has been used to develop mechanistically-based Quantitative Structure-Activity Relationship (QSAR) models that use reactivity descriptors to predict toxicity. In other studies, reactivity descriptors have been used as basis for grouping chemicals, with predictions of toxicity being obtained by read-across.

Chemical reactivity is generally determined by measuring the reaction rate or extent of reaction of a test chemical against a protein surrogate, which can be any type of nucleophile ranging from a small chemical, such as n-butylamine or propane thiol, through a protected amino acid, such as *N*-acetyl cysteine, to a synthetic peptide and even a protein such as human serum albumin (HSA).

The potential for using *in chemico* methodology in toxicology, including a set of recommendations for its further development, has been critically reviewed ~~by~~ (Cronin et al., 2009). The aim of this report is to give a more detailed review of *in chemico* studies published in the literature, with an emphasis on studies aimed at toxicity prediction. The review is intended to be illustrative rather than exhaustive, and as such is limited to abiotic (non-enzymatic) methods using small nucleophiles up to the size of peptides of less than 50 amino acids, as well as QSARs built from chemical reactivity descriptors.

2. Reactivity-based QSARs and read-across

QSARs have traditionally been built for structurally similar compounds or compounds belonging to the same family, e.g. benzene derivatives, ketones, or halogenated compounds. Commonly used descriptors have included the traditional Hammett (ρ) and Taft (σ^*) parameters. The former is an indicator of the susceptibility of the reaction rate with respect to the polar properties of the substituents, whereas the latter accounts for field and inductive properties. The two parameters allow for chemical reactivity prediction (Hammett, 1937; Taft, 1956). This approach has led to models with rather small applicability domains, which prevented QSARs from being a broadly applicable alternative methodology for toxicity prediction. The use of reaction-mechanism based applicability domains seems a more appropriate strategy, especially for predicting endpoints that are strongly related to chemical reactivity such as skin sensitisation. Compared to the classical functional group

based applicability domains, the reaction-mechanism based applicability domains can include different types of chemicals in the same domain, e.g. ketones and aldehydes. Reaction-mechanism based domains allow for more robust models because the fact that two molecules have the same functional group does not imply that they can perform the same reaction, which is in the end what defines the adduct that is formed and the toxic reaction. One of the advantages of this approach is that it allows for an easy classification of the compounds as it can be carried out systematically just searching for structural motifs. The concept of the QSAR applicability is explained elsewhere (Netzeva et al, 2005).

The reaction-mechanism based applicability domains were first used by Lipnick (Lipnick, 1991) to identify and rationalise outliers for a series of chemicals. Subsequently, Veith (Veith, 2004) proposed its use in order to make of QSAR a real alternative to experimental methods at the eyes of regulators, and Aptula and Roberts (Aptula & Roberts, 2006) brought the idea into practice using a classification purely based on organic reaction mechanisms and independent of the nature of the compounds. They defined 5 domains corresponding to Michael acceptors, SNAr electrophiles, SN2 electrophiles, Schiff base formers and acylating agents.

The assignment (Roberts et al., 2007a,b) of more than 90% of the chemicals of two skin sensitisation datasets (Ashby et al., 1995; Gerberick et al., 2005) of 106 and 211 compounds, respectively, showed that the classification of chemicals into the proposed reaction-mechanism based applicability domains was straightforward. A series of structural alerts were derived and coded (Enoch et al., 2008; Schultz, et al., 2007) into a simple computer-aided system. The system was tested with a dataset (Gerberick et al., 2005) that had previously been classified manually by experts (Roberts et al., 2007b) and consistent results were obtained although in some cases the compounds were classified into more than one reactivity domain. The authors proposed to rationalize these cases with the help of molecular orbital calculations, but in most of the cases it was concluded that the compounds were likely to react via different mechanisms. Thus, in order to properly classify the challenging cases it is necessary to carry out further experiments (Alvarez-Sanchez et al., 2004) so as to determine their main mode of action.

The concept of Relative Alkylation Index (RAI) was developed by Roberts and co-workers (Roberts et al., 1991; Roberts & Williams, 1982) to quantify the reactivity (alkylation potency) of chemicals. It is expressed as function of dose (D), chemical reactivity (K_{rel}), and lipophilicity (P):

$$RAI = \log D + A\log K_{rel} + B\log P \qquad \textbf{(I)}$$

where the constants *A* and *B* are determined experimentally and are only valid for the compounds within the same reaction mechanism, *i.e.* the constants are applicability domain dependent. The lipophilicity is modelled by the octanol/water partition coefficient and can be obtained either theoretically or experimentally.

The RAI was initially obtained as the relative rate of a given chemical towards n-butylamine (a nucleophile model for lysine) with respect to the reactivity of a reference chemical (Roberts & Williams, 1982). The usefulness of the RAI index has been demonstrated in numerous studies (Barratt et al., 1994; Basketter & Roberts, 1990; Franot et al., 1994a,b; Patlewicz et al., 2003; Roberts, 1985, 1987; Roberts & Basketter 1990, 1997, 2000; Roberts & Benezra 1993; Roberts et al., 1991; Roberts & Patlewicz 2002; Roberts & Williams 1982; Roberts et al., 2007c; Roberts et al., 1999). In particular, the RAI has been shown to be a successful predictor of skin sensitisation. A quantitative model to predict skin sensitisation potency (EC3 of LLNA experiments) on the basis of RAI measurements has the following general form (Roberts et al., 2006):

$$pEC3 = a\log k + b\log P + c \qquad \textbf{(II)}$$

where logP corresponds to the octanol/water partition coefficient and k corresponds to the reaction rate, which can be also estimated using the Hammett and Taft constants (Roberts et al., 2007c) or other

methods such as the direct theoretical calculation of the reaction rate (Schwöbel et al., 2010; Verhaar et al., 1996).

Roberts et al. proposed (Roberts et al., 2006; Roberts et al., 2007b) the use of the term quantitative mechanistic modelling (QMM) to define this type of QSAR whose meaningful components are not chosen statistically but derived mechanistically. The convenience of using such descriptors for the prediction of skin sensitisation potential is explained by Roberts et al. (Roberts & Aptula, 2008). These QSARs have been well characterized according to the OECD validation principles (OECD, 2007), and thus may be suitable for regulatory use (Roberts et al., 2007a).

In addition to the use of reactivity descriptors in QSARs, they have been proposed for use in read-across. For example, Roberts and co-workers (Roberts et al., 2008) illustrate how quantitative kinetic measures of reactivity can be applied to perform mechanism-based read-across of skin sensitisation potential. They emphasise the need for a chemical reactivity database which in addition to kinetic measures of reactivity includes other important parameters such as hydrophobicity (logP). In another study, Schultz and co-workers (Schultz et al., 2009) showed that the read-across approach can be used to rank qualitatively the skin sensitisation potential of an untested carbonyl-containing Michael acceptor chemical by using subcategories. Fifty compounds previously evaluated in the LLNA were placed in 10 subcategories defined by their polarized alpha, beta-unsaturated substructure.

3. Overview of *in chemico* applications

Schultz and co-workers (Schultz et al. 2006a) developed a framework and exemplified in which way *in chemico* experiments can be used to predict aquatic toxicity, hepatocyte cytotoxicity, and skin sensitisation. The requirements for a wide use of *in chemico* approaches are: a clear identification of the relation between electro(nucleo)philic reactions and toxicity, and the availability of quantitative *in vivo* data to be used as a reference for the reactivity experiments. In this sense, the possibility of using chemical reactivity as an alternative methodology has been especially important since the appearance of the LLNA, which provides quantitative data that can be used with the nucleophile depletion or adducts formation rates of chemical reactivity to generate QSARs. The determination of the reaction rates must be carried out carefully (Alvarez-Sánchez et al., 2003; Meschkat et al., 2001a,b) because of the possible presence of side reactions such nucleophile oxidations that can bias the real value.

3.1. Skin sensitisation

The skin sensitisation process starts with haptenation, the covalent reaction that takes place between a small chemical and proteins and which results in the chemical being incorporated into the structure of the proteins. Some authors (Aptula et al, 2007b) have argued that the term “hapten” is not precise enough and that it should be avoided in favour of electrophile. Nevertheless, this initial reaction is considered the Molecular Initiating Event in skin sensitisation, and forms the basis of most QSAR models and expert systems, as reviewed elsewhere (Patlewicz et al., 2007; Patlewicz & Worth, 2008). The state-of-the art of non-animal methods for skin sensitisation has been reviewed elsewhere (Vandbriel & van Loveren, 2010; Adler et al, 2011).

Skin sensitisation potential can be measured by means of the murine Local Lymph Node Assay (LLNA; Kimber and Basketter 1992).The LLNA is less invasive for animals than the traditional Guinea-Pig Maximisation Test (GPMT; Magnusson & Kligman, 1970) and the Buehler occluded patch test (Buehler, 1965). The sensitisation produced by the chemical is expressed as the Stimulation Index (SI), a measure of the proliferation of cells in draining lymph nodes of exposed with respect to the response in control animals. It is generally accepted (Basketter et al., 1999) that a sensitiser is a chemical that produces an SI above 3, that is the proliferation of T-cells in exposed animals is 3 times that of the control animals. The LLNA can be used to quantify the potency of a sensitiser by determining the Effect Concentration 3 (EC3), which is the concentration of test chemical that causes a SI of 3. EC3 is a very convenient way of quantifying skin sensitization since it allows for a comparison

of the toxic potency of chemicals and makes of LLNA a reference assay (Basketter et al., 1992) for the development of alternative methods including QSARs.

The relationship between reactivity and skin sensitisation has been reviewed by Gerberick and collaborators (Gerberick et al, 2008). Probably the first systematic study was by Landsteiner and Jacobs in 1936 (Landsteiner & Jacobs, 1936) where they studied the skin sensitisation properties of a series of nitrobenzene derivatives on guinea pigs. They observed a significant correlation between the sensitisation properties of chemicals and reactivity with aniline. Different types of nucleophiles have been used for the experiments since then. For instance, Liberato and co-workers (Liberato et al., 1981) used (S)-N-acetylcysteine and 1-aminopentane as models of nucleophilic sites of serum albumin to study the reactivity patterns of the quinine derivates of urushiol (poison oak/ivy cathecols). Also, cadaverine and derivatives were used as models for lysine residues (Osamura et al., 1981; Sekine & Unno, 1988). In the last work, the authors went one step forward and studied the formation of adducts by thin layer chromatography and liquid chromatography.

The use of small chemicals as nucleophiles was progressively abandoned in favour of small synthetic peptides. This seems conceptually more appropriate since peptides are more similar to proteins, although they cannot mimic their exact 3D structure. Wass and Belin (Wass & Belin, 1990) used synthetic peptides to predict the sensitisation potential of inhaled chemicals. The authors used three peptides containing lysine and tyrosine. The former amino acid was chosen because it had been previously shown (Pepys, 1986; Tse & Pesce 1979) that isocyanates, ethylene oxide, and anhydrides reacted with the lysine residues of proteins. On the other hand, tyrosine was chosen because it absorbs UV light and could be easily detected in high-performance liquid chromatography (HPLC).

As mentioned above, not only the nucleophile plays a crucial role in *in chemico* approaches but also the reaction conditions. In the skin sensitisation endpoint this factor is especially important because the skin has a pH of 5.5, but the physiological pH is 7.4. The skin sensitisation process is mainly dependent on the ability of the chemical to cross the stratum and also to bind to the proteins once it has been internalized. Thus, the pH variation during internalisation is a challenge for the *in chemico* methods as it strongly affects the nucleophile's reactivity. Ahlfors et al. (Ahlfors et al., 2005) studied the ability of hexahydrophtalic anhydride (HHPA), a compound used in the manufacture of plastics, to form adducts with different amino acids at skin and physiological pH. They tried to explain the various allergic effects that it causes, i.e. rhinitis, conjunctivitis, urticaria, asthma type I (mainly caused by reactions to lysine), and allergic contact dermatitis type IV (mainly caused by reactions to cysteine) (Ahlfors et al., 2003). For this purpose, the authors used a number of amino acids and synthetic peptides to determine if HHPA was capable of performing multiple reactions. The results showed that only adducts with cysteine and proline were formed at skin pH (5.5). Surprisingly, most of the formed adducts took place with proline, although cysteine and lysine were also found to be reactive. More adducts were detected at physiological conditions (pH 7.4) since reactions with cysteine, histidine, lysine and tyrosine were detected.

Although the use of synthetic peptides has been generalized in the last decade Meschkat et al. (Meschkat et al., 2001a,b; Roberts et al., 2007c) showed that small chemicals could be a perfect complement for peptide reactivity, especially to determine reaction mechanisms in complicated cases. They used imidazole, butylamine, phenol (sodium phenoxide), phenolate, propanethiolate and N-Acetyl-Cys to study the different skin sensitisation potential of alkane and alkenesultones, and Alvarez-Sanchez and co-workers (Alvarez-Sánchez et al., 2003) for two types of isothiazolones. The rationalization of the results was not easy in the former case because the authors could not find strong evidences of the higher sensitisation of alkenesultones with respect to alkanesultones. They concluded that the different potency was caused by the combination of selective reactivity of alkenesultones to amino groups and the fast hydrolisation of alkanesultones. On the other hand, the models clearly explained the higher sensitisation potential of 5-Chloro-2-methylisothiazol-3-one (MCI) with respect to 2-Methylisothiazol-3-one (MI), since MCI showed higher reactivity to nucleophiles and faster reactions with thiol groups. Further evidences of this were obtained later (Alvarez-Sanchez et al., 2004) with the help of GSH and a synthetic peptide analogous to the N-terminal chain of globin, and

explained theoretically by Aptula and co-workers (Aptula et al., 2005) In addition, they provided a skin sensitisation potency prediction tool, named the Activation Energy Index (AEI). It was defined as the sum of the Highest Occupied Molecular Orbital (HOMO) and HOMO-1 energy differences between the reactants (thiazolones and nucleophiles). It was shown that the AEI could rank the reactivity of isothiazolones with the different nucleophiles correctly, and therefore could successfully qualitatively predict their skin sensitisation potential.

The use of peptide reactivity assays has been generalised since the work of Gerberick and co-workers (Gerberick, et al., 2004). They developed a peptide reactivity assay which used different peptides containing cysteine, lysine, histidine and glutathione (GSH). GSH is a cysteine containing tripeptide that is the most abundant peptide in cells and acts as antioxidant and cell protector thanks to its high reactivity to electrophiles. In addition, it is known that low levels of GSH are related to important diseases such as cancer, asthma, Alzheimer, and Parkinson. The reactivity assay developed by Gerberick and co-workers was based on the use of GSH depletion rates to classify the test chemicals into four categories, which consisted in: Weak, Moderate, Strong, and Extreme sensitisers. The depletion rates were compared to the EC3 values of LLNA assays, and despite a significant correlation between the depletion rates and EC3 was observed, the peptide binding assays did not show enough accuracy to clearly classify the chemicals in the four categories. This work, however, was later extended (Gerberick et al., 2007) to 82 chemicals and various assays with different peptide-test compounds ratios were performed. Different models were built using the different assays, and a significant improvement with respect to the first approach was achieved. The best results were obtained with a model with 5 components: GSH, Cys (1:10 and 1:50), and Lys (1:10 and 1:50). With this model, 94% of the compounds were correctly classified and only 5 were misclassified. A very good compromise between results accuracy and experimental complexity was obtained with a model which only included assays with two peptides, cysteine (1:10) and lysine (1:50). With such a model, 89% of the compounds were correctly classified, and only 9 were misclassified.

Although the limitations of these types of experiments are important because they cannot account for aspects such as skin penetration, water solubility, metabolism transformations, and immune recognition; they were found to be able to distinguish between weak and strong sensitisers, and therefore proved to be useful as a screening tool. The authors did not envisage this assay as a replacement for the LLNA but as a component of a multi-test, which would also take into account metabolism and solubility reducing the dependence on the *in vivo* experiments.

Natsch and co-workers (Natsch et al. 2007) applied a similar assay to a series of fragrance molecules and went one step further by using dose-response curves instead of fixed times, analyzing formed adducts by LC/MS, and using other peptides derived from proteins such as Cor1C-420, known to be reactive with the proposed chemicals. The use of CorlC-420 was found to be very convenient because of its high reactivity, high solubility, and no formation of precipitates. In general, the new method was highly reproducible and performed very well with strong and moderate sensitisers although some errors prompted with weak allergens. The errors were mainly detected for aldehydes, which the LC/MS showed to be due to peptide oxidation. Thus, the depletion curves of aldehydes used to determine the skin sensitisation potency were not caused by reactions with the test compounds but to auto-oxidation. In general, the assay was found not to be able to classify non α,β-unsaturated aldehydes, but the modifications included proved essential for the understanding of the peptide reactivity and helped to improve the toxicity estimation. This method was subsequently improved by the same authors (Natsch & Gfeller, 2008) and allowed to determine simultaneously the peptide depletion, peptide oxidation, adduct formation, and thiol reactivity. The result was a more demanding experiment but which provided a set of tools for a better prediction of skin sensitisation potential.

The evolution of these methods resulted in the high throughput kinetic profiling approach (Roberts & Natsch, 2009). This method measures the depletion rates of the heptapeptide Cor1C-420 (Gerberick et al., 2004) (Ac-RFAACAA) at different reaction times and with different initial concentrations, giving a matrix of depletion rates. The authors compared their results with previous ones which had been obtained with methods that calculated the reaction rates at a fixed time rather than performing a full

kinetic profiling. Surprisingly, the theoretically less accurate experiments showed good correlation with the full kinetic profiling. However, the latter methodology is more robust as it does not suffer from drowning out effects caused by low solubility of the test compounds, which may affect the determination of the reaction rates. The authors used the kinetic profiles of 27 substances and the pEC3 values of LLNA experiments to generate a quantitative mechanistic model for predicting the skin sensitisation properties of Michael acceptors. The model only included 10 compounds as 4 outliers were discarded (the rest of compounds were found not to belong to the Michael acceptor domain). In spite of the rather low number of compounds included in the model, it was the first to contain various activation groups such as aldehydes, ketones, esters, and pyridines. It was surprising that the inclusion of the logP term did not improve the model, denoting that the toxicity was mainly driven by chemical reactivity. The model read as:

$$pEC3 = 0.24(\pm 0.04)\log K + 2.11(\pm 0.24) \qquad \textbf{(III)}$$

n=10, r^2=0.836, s=0.11, and F=40.8

Aleksic and co-workers (Aleksic et al., 2009) also tried to improve the existing peptide binding assays by modifying them so as to obtain the maximum information possible. They proposed to analyze the reactivity of chemicals with seven peptides, six of them of generic sequence Ac-FAAXAA, where X corresponded to the amino acids cysteine, histidine, alanine, lysine, arginine, and tyrosine, and another one with an N-terminal nucleophile, namely FAAAAAA. The reaction conditions were optimized and the pH was set closer to the pKa value of the nucleophile so as to maximize the reactivity of the nucleophiles and widen their applicability domain. The authors chose 36 chemicals of various sensitising potencies and determined their chemical reactivity by measuring peptide depletion rates. The results showed that EC3 strongly correlated with the depletion rates of the Lys, Cys, and N-term containing peptides, but not with the peptides containing other amino acids. The authors observed that the Cys and N-term depletion rates correlated to Lys but not between them. This finding pointed out that the two peptides were probably describing different sensitisation processes. Analysing the data for the different peptides it was observed that only Lys depletion was able to predict sensitisation, because although Cys could differentiate classes of sensitisers, some non-sensitisers were found to react with it. Taking Cys and His depletion values together, a rule of thumb could be derived for predicting sensitisation: If a chemical was able to deplete His over 40% it was likely to be an extreme sensitiser and if it depleted Cys below 50%, it was likely to be a weak or non-sensitiser. If the chemical depleted Cys more than 50% and His less than 40% it fell on a blurry domain for which the prediction was not reliable. The large amount of generated data increased the accuracy of the method since it allowed for a double check of the depletion rates. For instance, in the cases for which the peptide depletion was not confirmed to be caused by reaction with the test chemical, MS analyses were carried out to determine the nature of the formed adducts and the reaction mechanism. Thank to this technique, it was possible to detect in many cases that the depletion was caused by peptide-peptide reaction, or that multiple adducts and different sequential reactions had taken place. It is worth to mention a case in which the reaction between His and benzyl bromide was found to stop before the consumption of all benzyl bromide. It was concluded that the reaction was competitive with hydrolysis and also that it was inhibited by the sudden pH drop triggered by the formation of hydrogen bromide upon reaction with the peptide. The authors also tried to characterize the sensitisation potency of chemicals taking into account the depletion values of the six reactive peptides by means of hierarchical clustering, a method (Han & Kamber, 2001) that defines groups of data according to their similarity. Three groups consisting of non-sensitisers, weak sensitisers, and strong sensitisers were defined. All groups contained misclassified compounds and although most of them could be rationalised with the help of the generated data, the grouping showed too high uncertainty so as to be considered a good alternative. All in all, the authors presented a rather simple and flexible method for predicting skin sensitisation potency, which generates a vast amount of data that increases the quality of the predictions and that allows for an easy rationalisation of outliers. The challenge is to interpret these data and use it efficiently to improve toxicity predictions.

Aptula and co-workers (Aptula et al., 2005b) used the GSH reactivity assays (thiol reactivity) proposed by Schultz and colleagues (Schultz et al., 2005) and the TETRATOX (Schultz, 1997) (population growth impairment of Tetrahymena pyriformis). The depletion of GSH was measured as EC50, concentration of test chemical which gives a 50% depletion of free thiol under standard conditions, and the TETRATOX results were obtained as IGC50, which corresponded to the concentration of test chemical that inhibited the growth of Tetrahymena pyriformis by 50% and was calculated as the difference between the TETRATOX pIGC50 and the pIGC50 obtained from the narcosis equation, which was only dependent on logP. The two assays were used to predict the skin sensitisation potency (Gerberick et al., 2004) of 24 chemicals that covered all the sensitiser's categories, i.e. weak, moderate, strong, extreme, and non-sensitisers; and various reaction mechanisms such as Michael addition, pro-Michael addition, SNAr, SN2, acylation and Schiff base formers. The best model that could be obtained with GSH was able to distinguish sensitisers from non-sensitisers by defining the formers as chemicals with a pEC50 > -0.55. If the results were combined with TETRATOX, which defined the skin sensitisers as chemicals with an excess toxicity >0.50, the prediction improved substantially and the model was able to correctly describe 23 out of 24 compounds. GSH depletion rates were also used (Aptula et al., 2007a) for a series of compounds only belonging to the Michael acceptor domain but no direct correlation was found to LLNA.

As mentioned above, GSH was also used by Alvarez-Sanchez et al. (Alvarez-Sanchez et al., 2004) together with a synthetic peptide analogous to the N-terminal chain of globin (H2N-VLSPADKTNWGHEYRMFQIG-CO2H) to complete the work on MCI and MI started with small nucleophiles (Alvarez-Sánchez et al., 2003). The two compounds were found reactive against GSH, but they showed different reactivity with the model peptide. MCI, a strong sensitiser, was found to react with histidine and lysine by forming stable adducts, however, no adducts were formed with MI. This was in agreement with the previous work, but the use of the synthetic peptide with various amino acids confirmed that the different reactivity against amino acids (Mutschler et al., 2009) other than cysteine, caused the different skin sensitisation potential of the two compounds.

The same peptide was used in conjunction with LC-MS and MS/MS by Eilstein and co-workers (Eilstein et al., 2008) to study the reactivity of p-amino aromatic compounds. They used a p-amino surrogate, 2,5-dimethyl-p-benzoquinonediimine, which was found to react specifically with the ε-NH2 group of lysine forming a Schiff base adduct. The lysine adduct was not stable and the peptide was found to react further by deaminating lysine, which caused subsequent intermolecular cyclisations. It is worth mentioning that the lack of cysteine in the experiment introduces a high uncertainty on the mechanistic results since it is more reactive than the used amino acids, which would surely have changed the outcome of the experiment.

The same peptide but including a cysteine residue, namely DS3 (VLSPADKTNWGHEYRMFCQIG) was used (Jenkinson et al., 2009) to study the binding sites, reactivity, and lymphocyte proliferation of 2,5-dimethyl-p-benzoquinonediamine and a less substituted p-phenylamine, namely p-phenylendiamine (PPD). This was an especially challenging case because PPD and 2,5-dimethyl-p-benzoquinonediamine are in principle non-sensitisers, but in water and under physiological conditions they easily oxidize to benzoquinonediimines, which are chemically reactive (Eilstein et al., 2006, 2007). Thus, these compounds were pre-haptens. The results showed that only adducts with cysteine residues were observed when the p-amines were incubated with the model peptide. Surprisingly, when PPD was incubated solely with N-acetyl cysteine, N-acetyl lysine or GSH, no adducts were detected. The presence of the amino acids seemed to inhibit the oxidation of PPD. This was confirmed when DS3 and PPD were incubated together with and without GSH, and a decrease on the formation of PPD-DS3 adducts was observed in the former case. Thus, the different reactivity of PPD against the different amino acids reported by Aleksic and co-workers (Aleksic et al., 2009) is explained by the different pH conditions of the experiments and the importance of the micro environment created by the residues nearby the nucleophilic centres of the residues. This last effect must be especially important in the DS3 case since due to its size it may generate a considerably complex environment that can affect the pKa of the nucleophilic centres and their reactivity.

The same procedure was applied (Fleischel et al., 2009) to study phenyl isocyanate and p-tolyl isocyanate, surrogates of industrially used aryl isocyanates that were found to be strong respiratory and skin allergens in animal models. The authors marked both compounds with 13C and used nuclear magnetic resonance (NMR) to determine the formation of adducts upon reaction with nucleophiles. The studies were carried out in solvent and pH conditions which minimised hydrolysis. The reactions against the amino acids of the form N-Ac-X (where X corresponds to Lys, His, Arg, Trp, Ser, Tyr, Thr, Cys, and Met) showed that the isocyanates were only reactive to Cys and Lys, even after 40 days of exposure, being Cys the most reactive one. Accordingly, adducts derived from reactions with amino and thiol groups were observed after incubation of the isocyanates with GSH or the model peptide H2N-VLSPADKTNWGHEYRMFQIG-CO2H, which corresponds to DS3 but without the Cys residue.

The NMR technique was used by Fujisawa and Kadoma (Fujisawa & Kadoma, 2009) to develop some QSARs for acrylates and methacrylates, which are used in dental resins. The authors used the chemical shifts (δ) of the atoms of the α,β-unsaturated moiety, namely C_α, C_β, H_a, and H_b (where the hydrogens correspond to the ones attached to the C_β) to predict the reaction rates of (meth)acrylates against GSH. Various QSARs using δ_{C_β}, δ_{H_a}, and δ_{H_b} as descriptors showed strong correlations to logK:

$$\log K_{GSH} = -48.6(\pm 0.2) + 0.4(\pm 0.0)\delta_{C_\beta} \qquad \textbf{(IV)}$$

n=11, r^2=0.998, p<0.001

This finding is explained by the fact that acrylates react via Michael addition reaction type, which is driven by the reactivity (electrophilicity) of C_β, which is strongly related to the chemical shift. Although the domain is small and the compounds are very similar, the high correlation between logK and δ is surprising. On the other hand, the logK of (meth)acrylates showed no correlation to acute toxicity in mice, as it had been seen elsewhere (Tanii & Hashimoto, 1982), and only when logP was included in the model a significant correlation was obtained:

$$LD_{50} = 15.1(\pm 17.3) + 37.9(\pm 10.1)\log P - 16.4(\pm 6.7)\log K \qquad \textbf{(V)}$$

n=8, r^2=0.88, p<0.01

However, the validity of this model is dubious due to the high variability of the coefficients and the small number of points. In addition, Tanii and Hashimoto (Tanii & Hashimoto 1982) showed that logP and logK were highly dependent in (meth)acrylates and they could only develop models with one descriptor. They found high correlations between these descriptors and the oral toxicity of mice (LD50) to acrylates,

$$\log\left(\frac{1}{LD_{50}}\right) = -0.423\log P - 0.735 \qquad \textbf{(VI)}$$

n=6, r^2=-0.9909, P<0.05

and also log*K*,

$$\log\left(\frac{1}{LD_{50}}\right) = 1.808\log K - 4.092 \qquad \textbf{(VII)}$$

n=8, r^2=0.889, P<0.05

but not for methacrylates, which showed correlations of -0.795 and 0.438 respectively. However, more chemicals must be included in the models in order to consider them for being used in risk assessment.

A slight variation of the methods reported so far for skin sensitisation prediction is the Surface Plasmon Resonance (SPR) (Nguyen et al., 2007). The SPR assay consists on optical biosensors that detect the intensity of the light reflected on a thin gold film placed between two layers of different refractive indexes. One molecule (in this case a protein surrogate) is immobilized on the surface and the others (allergens to be screened) are flowed continuously over the surface. When a molecule of the flow binds to the surface, therefore interacts with one of the immobilized molecules, the refractivity index of the layer changes, which varies the intensity of the reflected light. The changes in intensity are measured in real-time by the biosensors and are nearly proportional to the change of mass near the surface. This allows for the determination of the bound molecules, their concentration, and their binding and dissociation kinetics. The levels of interaction are measured by arbitrary units named resonance units. As in the classical *in chemico* experiments, the predictive power of this assay relays on the degree of accuracy between the skin sensitisation potency of the flowed chemicals and their binding affinity to the immobilized target(s).

Achilleos and co-workers (Achilleos et al., 2009) applied the SPR to 21 chemicals and 17 fragrances of different skin sensitisation potential, using as immobilized molecules the most reactive amino acids, i.e. cysteine, lysine, and histidine. The authors observed that the weak sensitisers established weak interactions with the targets and that they could be washed off by the flow. Moderate sensitisers were found to only establish strong interactions with cysteine, while strong and extreme sensitisers bound strongly to the different amino acids. This methodology offers fast and simple screening capabilities since it only requires low concentrations of chemicals, and no chemical labelling. Due to its novelty, it needs further experimentation to be used in regular risk assessments and its capability of testing mixtures of compounds needs to be assessed. Unfortunately, as most of the *in chemico* approaches it can neither account for pre- or pro-haptens.

By using 4-nitrobenzenethiol (NBT), Chipinda et al. (Chipinda et al., 2010) became one of the few exceptions that have not used peptides as protein surrogates in the last decade. They proposed a fast and simple stopped-flow and UV spectrophotometric assay which allowed for the determination of a wide range of reaction rates of chemicals. The method is especially convenient for studying chemicals which exhibit very fast reaction rates (t1/2=0.4ms to t1/2=46.2s) and are problematic in regular peptide reactivity assays due to their low sensitivity. With this method the drowning-out effects can be easily eliminated by changing the solvent. There are no evaporation losses of test chemical thanks to the use of a closed cell as reaction chamber. Moreover, the side reaction effects can be eliminated by carrying out the experiments with different electrophile concentrations and obtaining the reaction rate from the fitting of the different initial reaction rates to the concentration of electrophile. The authors tested 23 chemicals which were classified into Michael acceptors, SN1/SN2, and Acylating Agents applicability domains. The reaction rates obtained for the chemicals were correlated with the pEC3 values of corresponding LLNA experiments. Correlations of 0.87, 0.96, and 0.93 were found for the Michael acceptor, SN1/SN2, and Acylating Agent domains respectively, although the last two domains were not representative as they only contained 6 and 3 compounds each. Nevertheless, although low, a significant correlation of 0.74 was obtained when all the compounds were included in the same model. The models for the Michael addition and the general domain are presented next:

$$pEC3 = 0.81(\pm 0.11)\log K_a + 2.13(\pm 0.23) \qquad \textbf{(VIII)}$$

n=10, r^2=0.87, s=0.65, and F=52.3

$$pEC3 = 0.75(\pm 0.11)\log K_a + 1.79(\pm 0.21) \quad \textbf{(IX)}$$

n=19, r^2=0.74, s=0.71, and F=47.2

where logK_a corresponds to the reaction constant derived from the full kinetics.

3.2. Aquatic toxicity

A pioneering work for predicting aquatic toxicity by means of chemical reactivity was carried out by Hermens and co-workers (Hermens et al., 1985). They studied the toxicity to guppy (LD50) for 16 reactive organic halides and observed that the compounds that were reactive to 4-nitrobenzylpyridine (NBP) were significantly more toxic than predicted by narcotic QSARs. No correlation was found to hydrophobicity and the best fit for guppy lethality was obtained with a bilinear QSAR using chemical reactivity to NBP as descriptor:

$$\log \frac{1}{LC_{50}} = -1.30 \log\left(1.604 - \frac{1}{K_{NBP}}\right) + 4.35 \quad \textbf{(X)}$$

n=15, r^2=0.88, s=0.44

The work was completed (Hermens et al., 1987) with a series of organophosphorus compounds. As in the previous case, the inclusion of KNBP significantly improved the models, the best model being that which included hydrophobicity (π) and KNBP:

$$\log \frac{1}{LC_{50}} = 0.23(\pm 0.08)\sum \pi + 0.80(\pm 0.12)\log K_{NBP} + 2.77 \quad \textbf{(XI)}$$

n=9, r^2=0.92, s=0.19

The authors recognised the potential utility of the model and especially the KNBP assay, although it was only suitable for nucleophilic substitution reactions (SN) and not for other types like Schiff base formers or pro-electrophiles. In the same work, other QSARs for predicting LC50 and KNBP rates were built using different descriptors such as Hammett (σ) constants, hydrophobicity (π), and calculated KNBP. In all cases it was shown that KNBP was more important than hydrophobicity in order to obtain good toxicity predictions. On the other hand, hydrophobicity (logP) was found (Deneer, et al., 1988b) to be necessary to correctly predict the fish lethality of epoxides,

$$\log \frac{1}{LC_{50}} = 0.39(\pm 0.05)\log P + 3.0(\pm 0.4)\log K_{NBP} - 2.25 \quad \textbf{(XII)}$$

n=12, r^2=0.945, s=0.27

Purdy showed (Purdy, 1991) that the reactivity of a series of epoxides against NBP could be successfully predicted with a superdelocalizability parameter calculated for the orbitals of the epoxide carbon atoms along the epoxide oxygen bond. The author also showed that the fish toxicity (LC50 of Poecilia Reticulata) could be accurately predicted (r^2=0.91) using only the superdelocalizability and logP descriptors.

The role that hydrophobicity plays in fish toxicity seems to be highly dependent on the type of chemical studied, since logP was shown (Deneer et al., 1988a) to be the only descriptor needed to predict the fish lethality of aldehydes:

$$\log \frac{1}{LC_{50}} = 0.36(\pm 0.04)\log P - 2.54 \qquad \textbf{(XIII)}$$

n=14, r^2=0.923, s=0.19

Although in this case cysteine was used as nucleophile instead of NBP, the fact that hydrophobicity was sufficient for the fish lethality prediction indicated that the uptake of aldehydes is the limiting factor of the toxic process of aldehydes.

Freidig and co-workers (Freidig et al., 1999) tried to build QSPRs (Quantitative-Structure Property Relationship) for predicting the reactivity of acrylates and methacrylates against nucleophiles of different strength including water, hydroxyl anion, and GSH. Due to the too small amount of data, no QSPRs for the hydrolysis of acrylates could be generated. However, a linear free energy relationship for the hydrolysis of methacrylates showed that the hydrolysis rate could be significantly correlated to the Taft parameter (σ^*). The authors built a QSPR model for the prediction of GSH reactivity. It only included theoretical parameters such as charged densities on the carbon atoms involved in Michael type additions and E_{LUMO}:

$$\log K_{GSH} = 2.65q(C_\beta) - 1.37q(C_\alpha) + 3.39q(C_1) - 49.33E_{LUMO} \qquad \textbf{(XIV)}$$

n=12, r^2=0.932

The parameters were calculated at the *ab initio* and semiempirical levels, revealing that the former were a better correlation with GSH reactivity, which was translated into better reactivity predictions. *Ab initio* methods are based on the solution of the Schrödinger equation to obtain the molecular energy. No experimental parameters are used in the calculation. On the other hand, the semiempirical methods are also based on the solution of the Schrödinger equation but they make use of a series of parameters which are adjusted to experimental data in order to obtain the best results. The semiempirical methods are much faster than *ab initio* ones but they might perform poorly in some cases if the system of study is not properly described by the model (method or theory).

In another study, the same authors showed that the predictability of QSARs could be improved if the datasets were divided by modes of action differentiating reactive toxicity from narcotic toxicity (Freidig & Hermens, 2001).

Schultz and co-workers (Schultz et al., 2005b) used the GSH assay to predict the IGC_{50} of Tetrahymena pyriformis for a series of isothiocyanates. They used a model (Schultz & Netzeva, 2004) developed for substituted benzenes based on logK_{ow} and the maximum acceptor superdelocalizability (A_{max}).

$$\log\left(\frac{1}{IGC_{50}}\right) = 0.545\log K_{ow} + 16.21A_{\max} - 5.91 \qquad \textbf{(XV)}$$

n=384, r^2(pred)=0.856, s=0.275, F=1163, Pr > F=0.0001

Accordingly, good results for phenyl and naphthyl derivatives were obtained, although the good performance was partly due to the fact that all the compounds used in the model were Michael acceptors. However, it was surprising that the model performed better than the one which only included GSH reactivity as descriptor:

$$\log\left(\frac{1}{IGC_{50}}\right)=1.77\left(\log\frac{1}{EC_{50}}\right)+0.60 \qquad \textbf{(XVI)}$$

n=12, r^2=0.718, s=0.34, F=26, q^2=0.629

Both models overpredicted the toxicity of 4-butylphenyl, benzoyl, and cinnamyl analogues, probably due to hydrolisation. The authors concluded that benzyl-like derivatives were more toxic than phenyl-like ones because of the different electron density at the central C atom, which is the one attacked in the Michael-addition type reactions.

Another study (Schultz et al., 2006) performed by some of the authors showed a different model for aquatic toxicity (IGC50 of Tetrahymena pyriformis) for 12 Michael-type acceptors:

$$\log\left(\frac{1}{IGC_{50}}\right)=0.975\left(\log\frac{1}{EC_{50}}\right)-0.592 \qquad \textbf{(XVII)}$$

n=12, r^2=0.952, s=0.24, F=221, Pr>F = 0.0001

The work was extended later (Yarbrough & Schultz, 2007) to carbonyl compounds containing (α,β-) unsaturated groups, i.e. aliphatic esters, ketones, and aldehydes. 41 chemicals were included and a rather similar model with a slightly better fit was obtained, yet only consisting of GSH reactivity:

$$\log\left(\frac{1}{IGC_{50}}\right)=0.936(\pm 0.055)\left(\log\frac{1}{EC_{50}}\right)+0.508(\pm 0.064) \qquad \textbf{(XVIII)}$$

n=41, r^2=0.846, s=0.35, F=214, q^2=0.832

It was encouraging, for the future use of *in chemico* approaches in toxicological assessment, that the GSH assay was able to distinguish the compounds reacting by Michael-addition from the non-reactive ones.

Böhme et al. (Böhme et al., 2009) modified the GSH assay and measured second order reaction rates and pseudo-first order for the less reactive compounds. Also, the amount of GSH depletion caused by GSH oxidation was measured so as to obtain the real GSH depletion. They measured the reaction rates for 26 compounds acting as Michael acceptors including 15 α,β-unsaturated ketones, 9 acrylates (methacrylates and crotonates), and 2 propiolates. An excellent correlation of r^2=0.91 between the GSH depletion reaction rate (K_{GSH}) and acute aquatic toxicity (48h growth inhibition of Tetrhymena pyriformis, EC50) was found for the 26 compounds:

$$\log EC_{50} = -0.673(\pm 0.042)\log K_{GSH} - 2.877(\pm 0.067) \quad \textbf{(XIX)}$$

n=26, r^2=0.91, q^2_{CV}=0.89, rms=0.30, rms_{cv}=0.34, and $F_{1,24}$=257

where q^2_{CV} corresponds to the correlation coefficient in the leave-one-out cross validation, rms is the root mean square error of the calibration, and $F_{1,24}$ is the Fisher test value.

The SN2 domain was analyzed with GSH by Schultz and co-workers (Roberts et al., 2009; Schultz et al., 2006; Schultz et al., 2007). They measured the reactivity against GSH (RC50) for 60 haloaliphatic compounds. Since a large correlation between pRC50 and KGSH was found (r^2=0.98), as in the study by Böhme and colleagues (Böhme et al, 2009), the authors decided to use pRC50 as descriptor instead of KGSH. The 60 compounds were classified according to their reaction characteristics into: non-activated primary halides, halides activated by an unsaturated hydrocarbon, halides activated by an electron-withdrawing group, and compounds with a not clearly defined reaction mechanism. All the non-activated primary halides were found to be not reactive to GSH, which was explained by the fact that their toxicity was consistent with narcotic effects and not to chemical reactivity. A highly significant correlation was obtained for the halides activated by electron-withdrawing groups:

$$pIGC_{50} = 0.94(\pm 0.07)pRC_{50} + 1.34(\pm 0.07) \quad \textbf{(XX)}$$

n=22, r^2=0.889, s=0.27, and F=161

It was observed that the GSH measure underestimated the toxicity of the second group of compounds, halides activated by an unsaturated hydrocarbon. Thus, the real toxicity was found to be higher than that obtained with the equation presented above. This was explained by the possible hydrolysis and evaporation side reactions that could have taken place when performing the GSH assay. The generation of a model for the last group of compounds was unviable due to the homogeneity of the group. However, some of the compounds were found to be correctly predicted by the model showed above, and they were included in the domain leading to the following model:

$$pIGC_{50} = 0.99(\pm 0.05)pRC_{50} + 1.28(\pm 0.05) \quad \textbf{(XXI)}$$

n=31, r^2=0.936, s=0.25, and F=426

The mismatch between experimental and calculated toxicity for the rest of the compounds, which were not correctly predicted with this model, was easily rationalized in terms of substituent effects on reactivity.

GSH reactivity was used in a slightly different approach by Gagan and co-workers (Gagan et al., 2007). They studied the toxicity of a mixture of organic chemicals, which counter intuitively is in general close to dose-additive, *i.e.* total toxicity is equal to the sum of the parts, even though the toxicity of the different chemicals is induced at different sites. A mixture of soft electrophiles with the non polar narcotic 3-methyl-2-butanone (NPN) was tested for its aquatic toxicity by means of the Microtox protocol, in which the chemicals are put in contact in aqueous solution with fluorescent bacteria (Vibrio fischeri) and the changes on the luminescence are analyzed along time giving the

time-dependent toxicity. The GSH reactivity was used to determine the chemical reactivity of the individual electrophiles to later compare it with the mixture reactivity and to determine the mode of action. The results indicated that S_N2 agents could have more than one site of action. The fact that the NPN:S_N2 mixtures exhibited a toxicity lower to that predicted by the dose-addition model, indicated that part of the toxicity exhibited by the S_N2 electrophiles was due to narcotic effects. On the other hand, the toxic effects of S_NAr chemicals were fully time-dependent and the toxicity of NPN:S_NAr mixture was closer to dose-additive. This was explained by the fact that the reactivity of S_NAr compounds was limited to the thiol groups and NPN to lipid membranes. Thus, due to the different modes of action of NPN and S_NAr, the toxicities had dose-additive behaviour. A second part of this work (Dawson et al., 2008) studied the mixture toxicity for a series of Michael acceptors. They observed that there was a clear correlation between toxicity in the Microtox assay and GSH reactivity. Comparing the results of both assays for compounds alone, mixtures, and different times of exposure, it was concluded that the compounds with lower reactivity against GSH may have react with different active sites, *i.e.* proteins and cell membranes. Accordingly, the mixtures of such compounds were found to have a toxicity just approximately dose-additive, whereas the mixtures of compounds which exhibited high reactivity to GSH were found to be strictly dose-additive. Very similar results were also observed for a series of alpha-halogenated acetonitriles (Dawson et al., 2010).

A theoretical descriptor developed for skin sensitisation prediction, AEI, was used by Aptula and co-workers (Aptula et al., 2005b) to predict IGC_{50} of Tetrahymena pyriformis. They used N-butylamine as nucleophile model to study 40 polyhydroxybenzene derivatives, thought to be pro-Michael acceptors. The compounds that could not be oxidized to reactive quinones, *i.e.* meta-substituted hydroquinones, were excluded from the model. Actually, their toxicity was shown to be correctly predicted by hydrophobicity, indicating that they were not pro-electrophiles but acted as polar narcotics. A significant and good correlation for the remaining compounds was achieved with AEI:

$$pIGC_{50}(adj) = -0.49(\pm 0.06)AEI + 6.85(\pm 0.69) \qquad \textbf{(XXII)}$$

n=18, r^2=0.821, q^2=0.774, s=0.24, F=73

Note that pIGC50(adj) corresponds to pIGC50 adjusted according to the number of reactive centres. Thus, molecules with 2 reactive centres are adjusted by –log(2). In this case, the addition of logP to the equation did not improve the model significantly.

3.3. Hepatotoxicity

GSH plays an important role in the liver since cell death after exposure to quinones is preceded by GSH depletion (Bolton et al., 1992). However, only a few *in chemico* applications using GSH depletion for the prediction of hepatotoxicity have been reported.

One of the few studies was carried out by Chan and co-workers (Chan et al., 2008) who studied in detail the hepatotoxic properties of a series of substituted p-benzoquinones. They determined the cytotoxicity of p-benzoquinones on rat and human hepatocytes and built some QSARs based on physicochemical properties of the compounds. They used E_{LUMO}, E_{HOMO}, dipole moment, nucleophilic frontier density, logP, molar refractivity, electron reduction potential, and electrophilic reactivity expressed as GSH depletion rate. From a theoretical point of view, it was expected that the GSH reactivity could be predicted by E_{LUMO}, since it corresponds to the energy of the orbital where the electron shared by the nucleophile will be placed after the attack, in other words it is an indicator of the susceptibility of the chemical to be attacked by a nucleophile. Consequently, the authors found a significant correlation between GSH reactivity of p-benzoquinones and E_{LUMO}:

$$\log K_{GSH} = -18.38 - 16.78E_{LUMO} - 3.19(E_{LUMO})^2 \qquad \textbf{(XXIII)}$$

n=10, r^2=0.80, P=0.008

In addition, rat hepatotoxicity was found to be significantly correlated to GSH reactivity,

$$\log LC_{50} = 4.65 - 0.92 \log K_{GSH} \quad \textbf{(XXIV)}$$

n=10, r^2=0.82, P<0.001

and even more to E_{LUMO},

$$\log LC_{50} = 424.54 + 17.7 E_{LUMO} + 3.36 (E_{LUMO})^2 \quad \textbf{(XXV)}$$

n=9, r^2=0.90, P=0.002

It was surprising that human hepatotoxicity was not correlated at all to GSH reactivity (r^2=0.40, P=0.1) or E_{LUMO} (r^2=0.55, P=0.1). The authors also defined some structure-activity relationships, mainly based on the electron-donor character and steric hindrance properties of the substituents, which were in agreement with those reported by Schultz et al. (Schultz et al., 2007). It is worth noting that no correlation was found between cytotoxicity and logP, indicating that membrane permeation and metabolic enzyme interaction, factors for which logP accounts for, are not limiting factors for hepatotoxicity of p-benzoquinones.

One of the latest *in chemico* applications that has been presented is the use of GSH as a trapping agent in a mouse liver microsomal assay for the screening of pharmaceutical reactive metabolites (LeBlanc et al., 2010; Ma & Chan 2010). The formation of reactive drug metabolites is the main cause of drug-induced toxicity, and thus it is very important to assess such an issue in an early stage of drug development.

In this experiment four drug components known to produce reactive metabolites were incubated with liver enzymes and GSH-Br, and the adduct formation was analyzed by LC and MS. The use of GSH-Br was a success as it offered higher sensitivity and lower false positives rates than the regular GSH.

3.4. Respiratory toxicity

Due to the lack of a well-validated method for the determination of respiratory allergens and hence of reference data, very few studies have used *in chemico* assays to predict respiratory toxicity. One of the few applications was presented by Rothe and co-workers (Rothe et al., 2008) who used a standard peptide binding assay (Gerberick et al., 2007) with several respiratory allergens. The experiment was not informative since the chemicals showed different reactivity to the peptides with no clear pattern. The authors pointed out that the lack of consistent results might be due to side reactivity with water or to steric hindrance of the peptide, although we doubt of the latter reason as it has been used before for other applications with no such problems.

A better result was obtained by Schultz et al. (2006a) who developed a QSAR model based on the RAI and GSH reactivity for predicting inhalation toxicity of 19 chemicals. The model showed a good correlation although with a small number of chemicals. Six non toxic chemicals were discarded since they were found to be non reactive against GSH, and from the remaining chemicals, only those considered Michael-type acceptors (a total of ten compounds) were included, leading to:

$$\log RD_{50} = 0.598 (\log EC_{50}) + 1.03 \quad \textbf{(XXVI)}$$

n=10, r^2=0.846, s=0.31, F=44, Pr>F = 0.0001

Further references on the development of novel approaches for respiratory toxicity are provided elsewhere (Roggen et al., 2008).

3.5. Miscellaneous toxicological effects

A number of studies have investigated the relationship between reactivity and genotoxicity, although contradictory results were obtained. For instance, the alkylation rate constants of chemicals with NBP (Eder et al., 1980; Eder et al., 1982; Epstein et al. 1955; Hemminki & Falck, 1979; Hemminki et al., 1980; Hemminki et al., 1983a,b; Neudecker et al., 1980) showed that the alkylation potential correlated well with mutagenicity, and that the reaction mechanisms played a major role (Singer, 1976). However, other studies (Müller et al., 1998) pointed out that *in chemico* methodology was better used as an exploratory tool than a predictive tool, showing in some cases very limited predictability (McCarthy et al., 1994).

Schmidt et al. (Schmidt et al., 2007) used the reactivity against GSH to understand the performance of dimethyl fumarate (DMF), a substance used to treat Psoriasis, a chronic inflammatory skin disease (Gottlieb, 2005). Psoriasis has been effectively treated during decades with DMF, but their mode of action and pharmacokinetics are not fully understood. DMF is highly reactive and is administered orally, thus it is seems difficult that DMF reaches the blood stream unaltered. Schmidt et al. measured GSH reactivity at different pHs to analyze the products formation and reaction kinetics of DMF and methylhydrogen fumarate (MHF), a DMF metabolite with longer half life suspected of being the real active species (see original work for related references). MHF was found to be 30 times less reactive than DMF, which suggested that MHF would remain longer in the cells and therefore might be the active species. Another hypothesis was that the active species was in fact the adduct GSH-DMF. The authors studied the metabolites of these adducts, that is N-acetylcysteine derivates, and the possible products formed upon reaction with DMF. They observed that the products were very similar to the ones formed upon reaction with GSH and after some other experiments with MHF, it was concluded that the low reaction rate of MHF at physiological pH explained its presence in blood after administration, and that MHF may account for the anti-inflammatory effects because of its longer unaltered presence in the cell.

Harder et al. (Harder et al., 2003) analysed the toxic effects of a set of chemicals against Escherichia Coli to determine their mode of toxic action. The chemicals were classified into three groups corresponding to GSH depletion, DNA damage, and unspecific reactivity. QSARs were built for each group using GSH and 2'-deoxyguanosine depletion rates, respectively. The inclusion of hydrophobicity did not improve the models, showing that the toxicity limiting factor was chemical reactivity. The authors also measured the toxic effects of the compounds on higher organisms such as algae, daphnia, and fish; and significant correlations were obtained with Escherichia Coli although the domains were too small to be considered ready-to-use QSARs. Thus, the relative toxicity of chemicals against Escherichia Coli was shown to be a good toxicity indicator, yet not a predictor, for higher organisms. In addition, the authors proposed the use of the chemical reactivity against the different surrogates as indicator of mode of action, which could be used in the classification of chemicals.

A different type of toxicity than the ones mentioned above is that caused by metals. Metals do not bind covalently to nucleophiles, but complexate with electron rich centres of proteins or enzymes (O, N and S) changing their structure, and therefore their activity. Metal complexation is related to protein malfunction, which may translate into allergic type reactions, although the type of response may vary depending on the route of exposure, concentration, and metal type. This number of variables makes of metal toxicity a process that is not fully understood. One of the very few studies in which in chemico techniques are used to determine metal toxicity was carried out by Razmiafshari and co-workers. (Razmiafshari et al. 2001; Razmiafshari & Zawia, 2000) They used a peptide of 26 amino acids as a surrogate for zinc finger proteins, which coordinate to a Zn atom and regulate gene expression. The

authors studied the interactions of the surrogate with the metals Pb, Hg, Cd, and Ca with NMR. The first three atoms were shown to complexate with His and Cys residues, whereas Ca was not. These findings were in agreement with another work of the same authors (Razmiafshari & Zawia 2000) in which they showed that the metals which modified the DNA binding properties of the protein also modified the DNA binding properties of the peptide. Thus, it was proved that the peptide a good surrogate for the zinc finger protein. More information on metal ion toxicity can be found in a review by Martin (Martin, 2006).

3.6. Integrated testing strategies

Natsch and co-workers (Natsch et al., 2009) have used a battery of data from various existing methods to predict the skin sensitisation potency of 116 chemicals. They used Cys depletion rates, luciferase induction cell-line based assay (ARE) (Natsch & Emter 2008; Wang et al., 2006) calculated cLogP, and toxicology *in silico* predictions. In the original proposal (Jowsey et al., 2006) and the subsequent update (Basketter & Kimber, 2009) the results of the battery test were obtained as a multiplication of factors of its individuals giving a final score that defined the sensitisation potency categories. With such an approach, if any of the assays determined that a chemical was non-toxic, and therefore scored zero, the global score became zero too. This made the method highly specific as all the tested chemicals that were found toxic in the battery test, were also positive in the LLNA. However, this also translated into low sensitivity as many sensitisers were not detected because in one of the assays they were not found active, and therefore obtained an overall score of 0. This approach was immediately noted by Roberts and Patlewicz (Roberts & Patlewicz, 2010) as a classical QSAR. They justified the need to express it as such and to suppress some of its components like bioavailability. On the same line, Natsch and co-workers (Natsch et al., 2009) also proposed variations of the multiplication approach consisting of the use of average scores of experimental assays. The authors applied the average scores to 116 skin sensitisers, and observed that neither the TImes MEtabolism simulator (TIMES) skin sensitisation (TIMES SS) model nor cLogP significantly improved the correlation. A regression analysis including all the data showed that the best results were obtained with only two parameters, corresponding to Cys depletion and the antioxidant response element (ARE) EC 1.5 Score, which is the average concentration of chemical inducing 1.5 fold gene activity. The final model, which exhibited near 87% sensitivity, 81% specificity, 92% positive predictability, 70% negative predictability, and 85% accuracy reads as:

$$LLNA\ Class = 0.357 Cys\ Score + 0.391 EC\ 1.5\ Score \qquad \textbf{(XXVII)}$$

$s=0.896$, $F=183.76$, $p<0.0005$

Another example of the potential of the *in chemico* approach in test-battery experiments can be observed in the work of Bergström et al. (Bergström et al., 2007). They combined the use of some *in vitro* and *in chemico* approaches to determine the skin sensitisation potential of carvoxime, an α,β-unsaturated oxime. Carvoxime, chemically identified as (5R)-5-Isopropenyl-2-methyl-2-cyclohexene-1-one oxime, was incubated with mouse and human liver microsomes together with GSH. The detection of GSH conjugates proved that carvoxime was a pro-electrophilic compound or a pro-hapten. The reactivity of the formed metabolites was further analyzed using a model peptide for GSH, namely N-ACME which corresponds to N-acetyl-L-cysteine and contains the same nucleophile group as GSH. Due to the high reactivity observed, a new mechanism involving nitroso intermediates was proposed. The ability of the metabolites to bind to proteins was analyzed with another model peptide, namely PHCKRM (Pro-His-Cys-Lys-Arg-Met). This peptide had already been used (Nilsson et al., 2005) to study a very similar pro-electrophile namely (5R)-5-isopropenyl-2-methyl-1-methylene-2-cyclohexene, whose metabolites were found to bind to cysteine and lysine residues; and to classify epoxides with respect to their skin sensitisation potency (Niklasson et al., 2009). This peptide is a good

surrogate for proteins because it contains hard and soft nucleophiles and allows for the determination of the reactive sites. In addition to all the *in chemico* experiments, the toxicity of the metabolites was determined by means of the LLNA assay, and their cross-reactivity by the Mouse Ear Swelling Test (MEST) approach (Gad et al., 1986) which is based on the measurement of the thickness of the ear of mice before and after exposure to the test chemical.

Reactivity towards GSH (Kay & Murfitt, 1966) was also used in test-battery strategies, mainly consisting of *in vitro* tests, to determine the aquatic toxicity for a series of chemicals with different modes of action. (Nendza & Wenzel 2006; Wenzel et al., 1997) The GSH assay was basically used to determine alkylating agents. The EC50 values of the test compounds were obtained by measuring the free GSH with alloxan (5,6-dioxyuracil), which can be determined spectrophotometrically.

4. Advantages and limitations of the *in chemico* approach

The main advantage of the *in chemico* approach with respect to bioassays is its simplicity. It allows for a high control of the experimental conditions and a high reproducibility. Furthermore, chemical reactivity can be explained in terms of organic chemistry alone. The *in chemico* approach provides a relatively easy means of screening chemicals with different model nucleophiles, determining their reactivity potency and studying their reaction mechanisms. In addition, *in chemico* experiments are practical since they can be carried out with instruments that are commonly found in regular laboratories, such as a Liquid Chromatography-Mass Spectrometer (LC-MS).

The main source of error of *in chemico* experiments is the degree of mismatch in the behaviour of the nucleophile, i.e. protein surrogate, and the real system. A small chemical cannot completely represent the behaviour of a protein, since the nucleophilicity of a protein nucleophile is affected by other parts of the protein. In addition, the nucleophilicity of the surrogate can be modified by the non-physiological conditions under which the experiment is carried out, which thus represents another source of error. However, the consequence of these effects is reduced if the chemical reactivity potency is treated on a relative scale since the chemical measurements will be systematically biased, but they will keep their relative potencies.

Like most *in silico* and *in vitro* methods, *in chemico* assays cannot on their own account for pre- and pro-haptens.

While *in chemico* methods cannot represent standalone alternatives to *in vivo* tests, thanks to their simplicity and reliability, they can be useful components of integrated testing strategies (Vonk, et al., 2009).

5. Summary and Conclusions

The objectives of this review were to introduce the *in chemico* approach to toxicity prediction and give a broad overview of the different applications that have been explored. We also intended this report to be used as a handbook of *in chemico* QSAR models, and for this purpose a list of published QSARs including endpoints modelled, nucleophiles and electrophiles used, descriptors used, and a short description of each model is provided (Appendix 1).

In chemico approaches are based on the relationship between chemical reactivity and toxicity. This relationship is particularly well established in the case of skin sensitisation (Divkovic et al., 2005; Karlberg et al. 2008; Kimber et al. 2010; Lepoittevin; 2006; Lepoittevin et al., 1997; Saint-Mezard et al. 2004). In addition, a number of studies have developed the *in chemico* approach for the prediction of aquatic toxicity and hepatotoxicity. The list was completed with a few additional studies with miscellaneous applications including respiratory toxicity, metal toxicity, and genotoxicity.

Most of the reviewed studies built quantitative models for toxicity prediction based on chemical reactivity measurements. Some of these models not only used reaction rates as experimental descriptors, but also theoretical reactivity descriptors such E_{LUMO}, and other parameters such as hydrophobicity. Most of these models were derived using small numbers of chemicals, and usually belonging to the same family. This limits the general use of chemicalmethodology in hazard assessments. To partially overcome this limitation, the use of reaction-mechanism based applicability domains (Aptula et al 2006; Lipnick 1991; Veith 2004) which allow for larger and more heterogeneous domains, is a promising way forward.

In most of the reported applications, chemical reactivity is measured as rates of depletion or adduct formation. These measurements can present artifacts, thus, the use of analytical techniques such as LC/MS or NMR is indispensable to assure accurate measurements.

The degree of accuracy between chemical reactivity and toxicity is highly dependent on the type of nucleophile used as a protein surrogate. The first *in chemico* studies generally used small chemicals such as n-butylamine or NBP as surrogates, but the use of peptides has become more common in the last 20 years. This shifting to the use of peptides is conceptually appropriate because of the higher similarity with proteins, which are in most of the cases the target of the toxicant. It is not clear whether any single peptide should be the nucleophile of choice since various have been investigated but none appears to have performed systematically better than the others. GSH is the most commonly used surrogate, but it only accounts for cysteine residue in its structure. The fact that many toxic effects are caused by interactions with residues others than cysteine and that some studies (Gerberick et al. 2007; Natsch et al., 2007) have shown better predictivity when using different peptides, indicates that a selection of different (residue-containing) peptides might be useful.

In chemico methods are in general highly reproducible, technically fast and simple to apply, and provide accurate information regarding reaction rates, reaction sites, and adduct formation. In addition, *in chemico* experiments are amenable to automation, which increases their reliability and efficiency. On the other hand, the existing models are generally restricted to small domains and need to be challenged further to establish their applicability. *In chemico* models cannot on their own be used to evaluate pro- and pre-haptens, and their capacity to predict mixtures still needs to be confirmed. For these reasons, *in chemico* approaches should not be used as standalone alternatives to animal testing, but they are expected to be useful components in integrated testing strategies.

6. Acknowledgements and Disclaimer

Any conclusions and opinions expressed in this document are those of the authors as individual scientists and do not constitute an official position by the JRC or the European Commission. The authors are grateful to Dr Enrico Burello (JRC) for helpful comments on this work.

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8. Appendix 1. List of *in chemico* studies reviewed

Citation	Endpoint	Nucleophiles used	Electrophiles studied	QSAR equations	Descriptors	Other information
Franot et al., 1994a	Skin sensitisation	n-butylamine	Butyrolactones	Biological Response= $a(RAI_i) - b(RAI_i)^2 + c(RAI_c) + d$ Sulfonates probit (% response)= $2.24(RAI_i) - 0.26(RAI_i)^2 + 0.54(RAI_c) - 2.23$ Lactones probit (% response)= $2.85(RAI_i) - 0.24(RAI_i)^2 + 0.82(RAI_c) - 3.12$	Krel, logP, Dose	They also checked the Relative Elicitation Potential, to analyse the cross reactivity potential of chemicals.
Deneer et al., 1988a	Aquatic toxicity (Poecilia Reticulata)	Cysteine	Aldehydes	$-\log(LC_{50}) = (0.36 \pm 0.04)\log P - 2.54$ $-\log(LC_{50}) = (0.36 \pm 0.04)\log P - 2.54 - (0.08 \pm 0.05)\log(Kcys) - 2.32$	Log P and Kcys	Inclusion of K_{Cys} did not improve the QSARs
Deneer et al., 1988b	Aquatic toxicity (Poecilia Reticulata)	NBP	Epoxides	$-\log(LC_{50}) = (0.39 \pm 0.05)\log P + (3.0 \pm 0.04)\log(K_{NBP})$	log P and log K_{NBP}	
Verhaar et al., 1996	Aquatic toxicity (Poecilia Reticulata)	NBP	Organic halides	Various QSARs. Theoretical LC_{50} predictions were: $\log LC_{50} = (-0.51)\log Kow - (0.97)\log k_{NBP} - 1.80$ [n=12, r^2=0.63, Q2=0.39] $\log LC_{50} = (-0.61)\log Kow - (0.80)\log k_{NBP} - 1.13$ [n=5, r^2=0.94, Q2=-2.70]	Log $K_{o/w}$ theoretical and experimental, and logK_{NBP} theoretical and experimental.	Theoretical results very similar to the ones obtained with experimental descriptors.
Freidig et al., 1999	Aquatic toxicity	GSH, H_2O and OH	Acrylates and methacrylates	Hydrolysis of methacrylates (reaction with respect to methyl methacrylate ($K_{Bmethyl}$) $\log(K_B/K_{Bmethyl}) = 1.25(\pm 0.25)\sigma^* - (0.18 \pm 0.09)$ Rx with GS^- (reduced GSH): $\log K_{GSH} = +2.65C_\beta - 1.37\,qC\alpha + 3.39qC_1 - 49.33E_{LUMO}$	Hydrolysis: Taft constants (σ^* and E(s)),E(s) does not improve correlation. GSH: Charge densities ($q(C_i)$) of C in acidic part of molecule, E_{LUMO} of electrophiles,	QSPR to reproduce experimental reaction rates

Citation	Endpoint	Nucleophiles used	Electrophiles studied	QSAR equations	Descriptors	Other information
Hermens et al., 1987	Aquatic toxicity (Guppy)	NBP	Organophosphorus	$Log(1/LC_{50})=-1.30\log(1604+1/K_{NBP})+4.35$ [n=15, r^2=0.88, s=0.44] $\log\left(\frac{1}{LC_{50}}\right)=0.23(\pm 0.08)\sum\pi+0.80(\pm 0.12)\log(K_{NBP})+2.77$ [n=9, r^2=0.92, s=0.19]	Hammett (σ, σ^-) constants LogK$_{NBP}$, Hydrophobicity (π)	One of the first studies to predict fish toxicity with K$_{NBP}$
Purdy, 1991	Aquatic toxicity	NBP	Epoxides	$\log K_{NBP}=34.9S^{N}C_1^{A}+1.67S^{N}C_1^{B}-5.11$ [r^2=0.91, n=12, s=0.074] $\log LC_{50}=-0.36\log P+119S^{N}C_1^{A}-2.7S^{N}C_1^{B}+13.5$ [r^2=0.90, n=12, s=0.24]	Superdelocalizability (S^N), logP	Experimental data taken from Deneer et al 1988b.
Van der Aar et al., 1996	GSH reactivity	GSH and MeS	1-chloro-4-nitrobenzenes	Based Catalyzed reaction logKs=4.58±(0.47) σ_p – 5.26(±0.23 [n=8, r^2=0.90, s=0.233] GST 4,4-catalyzed reaction log k_{cat}=2.42(±0.99) σ_p - 0.20(±0.49) [*r*= 0.705, *s=0*.499, *n*=8] log k_{cat}/K_m=3.23(±0.72)σ_p - 3.01(±0.35) [r=0.877, *s*=0.362, *n*=8] Hammett constant split into F and R Based Catalyzed reaction logKs=3.82±(0.47)*F* + 12.26±(1.33)(C1 charge) – 7.38(±0.29) [n=8, r^2=0.989, s=0.155] GST 4,4-catalyzed reaction logK_{cat}/K_m=3.61±(0.94)*F* + *6*.46±(2.67)(C1 charge) – 4.42(±0.58) [n=8, r^2=0.927, s=0.310]	Hammett constatns σ_p, F and R (inductive and resonance), charge,	
Harder et al., 2003	Hepatotoxicity	GSH and 2'-deoxyguanosine	reactive organochlorines, epoxides, and compounds with an activated double bond: acrolein, isobutyl acrylate, 2-hydroxyethyl acrylate, ethylacrylate, acrylonitrile, acryl amide, benzyl chloride, 4-nitrobenzyl chloride, 2,3-dichloro-1-propene, trans-1,4-dichloro-2-butene, styrene oxide, 2-(4-nitro-phenyl)oxirane, (2,3-epoxypropyl)benzene, 1,2-epoxybutane, epichlorohydrin, 2-methyl-2-vinyloxirane	$\log EC_{50}\,Ecoli=-0.87(\pm 0.04)x\log(K_{GSH})+1.60(\pm 0.08)$ [n=6, r^2=0.99, F=440] $\log EC_{50}\,Ecoli=-1.34(\pm 0.18)x\log(K_{gua})-2.43(\pm 0.40)$ [n=6, r^2=0.93, F=52]	logK$_{GSH}$ and logK$_{gua}$	QSARs for hepatotixicity according to mode of action. Small applicability domains.

Citation	Endpoint	Nucleophiles used	Electrophiles studied	QSAR equations	Descriptors	Other information
Aptula et al., 2005	Skin sensitisation	Propanethiolate, n-butylamine,	5-Chloro-2-methylisothiazol-3-one (MCI), 2-methylisothiazol-3-one (MI)	$AEI = \Delta E_{HOMO-1} + \Delta E_{HOMO}$	E_{Homo} and E_{Homo-1}	Theoretical explanation of the reactivity of the two molecules. A model is provided. It uses molecular energies of reactants. The Activation Energy Index is only valid if the TS of the reaction is similar to reactants.
Schultz, et al., 2005b	Aquatic toxicity (Tetrahymena pyriformis)	GSH	Series of substituted isothiocyanates (only subsitutents specified) Phenyl, 2,6-Dimethylphenyl, 3,5-Dimethylphenyl, 4-Butylphenyl, Naphthyl, 5-Indanyl, 1,4-Phenylene, Benzyl, • -Methylbenzyl, • -Phenylbenzyl, 2-Phenylethyl, 3-Phenylpropyl, 1-Naphthalenemethyl, Benzoyl, Cinnamyl.	$\log\left(\frac{1}{IGC50}\right) = 1.77\left(\log\frac{1}{EC_{50}}\right) + 0.60$ n=12, r^2=0.718, s=0.34, F=26, q^2=0.629	GSH EC_{50}	A model to predict toxicity to Tetrahymena pyriformis only using GSH reactivity as descriptor. It offers a modest performance (r^2=0.718).
Aptula et al., 2005a	Aquatic toxicity (Tetrahymena pyriformis)	n-butylamine (theoretical)	3-methylcatechol, 4-methylcatechol, catechol, 4-chlorocatechol, 4-nitrocatechol, 2,3-dihydroxybenzaldehyde, 1,2,3-trihydroxybenzene, 1,2,4-trihydroxybenzene, hydroquinone, chlorohydroquinone, bromohydroquinone, tetrachlorohydroquinone, tetrafluorohydroquinone, phenylhydroquinone, 2,5-dihydroxybenzaldehyde, 2,5-dichlorohydroquinone, tetrachlorocatechol, 2-nitroresorcinol	$pIGC_{50}(adj) = -049(\pm0.06)AEI + 6.85(\pm0.69)$ n=18, r^2=0.821, q^2=0.774, s=0.24, F=73	AEI (activation energy índex)	The study relates toxicity towards Tetrahymena pyriformis of a series of polysubstituted hydroxybenzenes. Two domains were found, consisting of polar narcotics and pro-Michael acceptors. A QSAR with a single descriptor (log D and AEI) was developed for each group. In both cases the models showed a good performance, and the toxic effects as well as the outliers were explained by mechanistic organic chemistry principles.

Citation	Endpoint	Nucleophiles used	Electrophiles studied	QSAR equations	Descriptors	Other information
Aptula et al., 2006b	Skin sensitisation	GSH	p-Benzoquinone, 2-Hydroxyethyl acrylate, Cinnamic aldehyde, Benzylidene acetone, a-Hexyl cinnamic aldehyde, Coumarin, 2-Hydroxypropyl methylacrylate, 1,4-Hydroquinone, p-Phenylenediamine, 3-Methyl catechol, Isoeugenol, Eugenol, 1-Chloro-2,4-dinitrobenzene, 2,4-Dichloro-1-nitrobenzene, b-Propiolactone, Oxazolone , 3-Propylidenephthalide, Dihydrocoumarin, Trimellitic anhydride, Phthalic anhydride, Citral, p-Aminobenzoic acid, Cinnamic acid, Sodium lauryl	Not really a QSAR. The model states that if a compounds exhibits a GSH pEC_{50}>-0.55 it is likely to be a sensitiser. If combined with TETRATOX, $pIGC_{50TETRATOX} - pIGC_{50narcosis} > 0.50$ determines skin sensitiser	pEC_{50}, $pIGC_{50TETRATOX}$ and logP (for calculation of $pIGC_{50narcosis}$)	An *in vitro* prediction method for skin sensitisation is provided. It includes GSH reactivity (thiol reactivity) and TETRATOX assays. The method is capable of predicting 23 out of 24 compounds correctly.
Chan et al., 2008	Hepatotoxicity	GSH	p-benzoquionones: chloroanil, 2,5-dichloro-benzoquinone, 2-bromo-benzoquinone, 2-tert butyl-benzoquinone, 2-methyl-benzoquinone, p-benzoquinone, 2,6-dimethyl-benzoquinone, 2,6-dimethoxy-benzoquinone, 2,3,5-trimethyl-benzoquinone, 2,3,5,6-tetramethyl-benzoquinone	GSH reactivity: $\log k_{GSH} = -18.38 - 16.78E_{LUMO} - 3.19(E_{LUMO})^2$ n=10, r^2=0.80, P=0.008 Rat hepatotoxicity: $\log LC_{50} = 4.65 - 0.92 \log k_{GSH}$ n=10, r^2=0.82, P<0.001 $\log LC_{50} = 424.54 + 17.7E_{LUMO} + 3.36(E_{LUMO})^2$ n=9, r^2=0.90, P=0.002	E_{LUMO}, K_{GSH}	Good models for hepatotoxicity were obtained GSH depletion rates (k_{GSH}) and E_{LUMO}. Some other parameters such as E_{HOMO}, dipole moment, nucleophilic frontier density, logP, molar refractivity, and electron reduction potential were also used to build some QSARs but poorer correlations were obtained.
Schultz et al., 2006a	Acute aquatic toxicity (Tetrahymena pyriformis)	GSH	12 Michael-type acceptors	Aquatic toxicity: $\log\left(\frac{1}{IGC50}\right) = 0.975\left(\log\frac{1}{EC_{50}}\right) - 0.592$ n=12, r^2=0.952, s=0.24, F=221, Pr>F = 0.0001 Respiratory toxicity: $\log RD_{50} = 0.598(\log EC_{50}) + 1.03$ n=10, r^2=0.846, s=0.31, F=44, Pr>F = 0.0001	EC_{50}	

Citation	Endpoint	Nucleophiles used	Electrophiles studied	QSAR equations	Descriptors	Other information
Yarbrough & Schultz, 2007	Acute aquatic toxicity (Tetrahymena pyriformis)	GSH	41 Michael-type acceptors(α,β-unsaturated carbonyl compounds)	$\log\left(\frac{1}{IGC_{50}}\right) = 0.936(\pm 0.055)\left(\log\frac{1}{EC_{50}}\right) + 0.508(\pm 0.064)$ n=41, r^2=0.846, s=0.35, F=214, q^2=0.832	EC_{50}	
Bohme et al., 2009	Acute aquatic toxicity (Tetrahymena pyriformis)	GSH	26 Michael acceptors compounds including 15 α,β-unsaturated ketones, nine acrylates (including methacrylates and crotonates), and two propiolates	$\log EC_{50} = -0.673(\pm 0.042)\log k_{GSH} - 2.877(\pm 0.067)$ n=26, r^2=0.91, q^2_{CV}=0.89, rms=0.30, rms_{cv}=0.34, and $F_{1,24}$=257	k_{GSH}	A new chemoassay which takes into account the amount of GSH reacted by oxidation was presented. The assay allows for the determination of the reaction rate and is only applied to Michael acceptors. The results are correlated to EC_{50} of Tetrahymena pyriformis with excellent results.
Fujisawa & Kadoma, 2009	Acute toxicity in mice (LD_{50})	GSH	methyl acrylate, ethyl acrylate, *n*-butyl acrylate, isobutyl acrylate, hexyl acrylate, 2-ethylhexyl acrylate, methyl methacrylate, ethyl methacrylate, isopropyl methacrylate, *n*-butyl methacrylate, isobutyl methacrylate, *t*-butyl methacrylate, allyl methacrylate, 2-hydroxyethyl methacrylate, benzyl methacrylate, ethylene glycol dimethacrylate, triethylene glycol dimethacrylate, tetraethylene glycol dimethacrylate, and 2,2-bis[4-(2hydroxy-3-methacryloyloxypropoxy)phenyl]propane	$\log k_{GSH} = -48.6(\pm 0.2) + 0.4(\pm 0.0)\delta_{C_\beta}$ n=11, r^2=0.998, p<0.001 $LD50 = 15.1(\pm 17.3) + 37.9(\pm 10.1)\log P - 16.4(\pm 6.7)\log K$ n=8, r^2=0.88, p<0.01	Chemical shift of C_α, C_β, H_a, and H_b (α,β-unsaturated moiety), logK and logP.	The chemical shift of the α,β-unsaturated moiety of a series of (meth)acrylates was used to determine reactivity against GSH with excellent correlations. However, the GSH reactivity could not be successfully correlated to acute toxicity in mice (LD_{50}).

Citation	Endpoint	Nucleophiles used	Electrophiles studied	QSAR equations	Descriptors	Other information
Tanii & Hashimoto, 1982	Acute toxicity in mice (LD_{50})	GSH	Methyl acrylate, Ethyl acrylate, n-Butyl acrylate, Isobutyl acrylate, 2-Hydroxylethyi acrylate, 2-Hydroxylpropyl acrylate, Methyl methacryiate, Ethyl methacrylate, isopropyl methacrylate, n-Butyl methacrylate, lsobutyl methacryiate, tert-Butyl methacrylate, 2-Hydroxyethyl methacrylate, 2-Hydroxypropyl methacrylate	$\log\left(\frac{1}{LD_{50}}\right) = -0.423\log P - 0.735$ N=6, r^2=-0.9909, P<0.05 $\log\left(\frac{1}{LD_{50}}\right) = 1.808\log K - 4.092$ N=8, r^2=0.889, P<0.05	logP and logK	QSARs are generated for the oral acute toxicity (LD_{50}) of a series of acrylates and methacrylates. A general model could not be found and logP and logK were shown to be highly dependent. Acrylates are better correlated to LD_{50}, with the two models showing correlation coefficients above 0.88
Roberts & Natsch, 2009	Skin sensitisation	Cor1C-420	27 compounds, 14 corresponding to Michael acceptor domain. Check original study for a complete list.	$pEC3 = 0.24(\pm 0.04)\log K + 2.11(\pm 0.24)$ N=10, R^2=0.836, s=0.11, and F=40.8	logK	A high throughput kinetic profiling method for the determination of depletion rates is proposed. It is applied to a series of compounds and a quantitative mechanistic model for the prediction for skin sensitisation is proposed for the Michael acceptor domain.

Citation	Endpoint	Nucleophiles used	Electrophiles studied	QSAR equations	Descriptors	Other information
Chipinda, et al., 2010	Skin sensitisation	Nitrobenzenethiol (NBT)	23 compounds including Michael acceptors, SN1/SN2 reactors, and acylating agents	Michael addition $pEC3 = 0.81(\pm 0.11)\log K_a + 2.13(\pm 0.23)$ N=10, r2=0.87, s=0.65, and F=52.3 SN1/SN2 $pEC3 = 0.85(\pm 0.09)\log K_a + 0.92(\pm 0.16)$ N=6, r^2=096, s=0.20, and F=93.7 Acylating agents $pEC3 = 1.03(\pm 0.27)\log K_a + 1.69(\pm 0.27)$ N=3, r^2=0.93, s=0.27, and F=14.23 Altogether $pEC3 = 0.75(\pm 0.11)\log K_a + 1.79(\pm 0.21)$ N=19, r^2=0.74, s=0.71, and F=47.2	$\log K_a$	A new method for the determination of *in chemico* reactivity which uses Nitrobenzenethiol is presented. This method is simple and very fast. Their main advantages are its possibility to measure a wide range of reaction rates, it does not suffer from drowning out effects or test chemical evaporation problems, and that is capable of obtaining the reaction rates without the interference of side reactions.
Roberts et al., 2009	Aquatic toxicity (Tetrahymena pyriformis)	GSH	60 haloaliphatic compounds (see original study for further details)	Halides activated by electron-withdrawing groups $pIGC_{50} = 0.94(\pm 0.07)pRC_{50} + 1.34(\pm 0.07)$ n=22, r^2=0.889, s=0.27, and F=161 Halides activated by electron-withdrawing groups + 9 compounds with not clear reaction mechanism $pIGC_{50} = 0.99(\pm 0.05)pRC_{50} + 1.28(\pm 0.05)$ n=31, r^2=0.936, s=0.25, and F=426	pRC_{50}	A QSAR for S_N2 haloaliphatic compounds is presented. The set of compounds was grouped in reaction mechanism subdomains. GSH reactivity of halides activated by electron-withdrawing groups showed significant correlation with aquatic toxicity. However, the reactivity of halides activated by unsaturated hydrocarbon was shown to underestimate toxicity probably due to hydrolosis and evaporation.

Citation	Endpoint	Nucleophiles used	Electrophiles studied	QSAR equations	Descriptors	Other information
Hermens et al., 1985	Aquatic toxicity (guppy)	NBP	Organic halides	$\log\frac{1}{LC_{50}} = -1.30\log\left(1.604 - \frac{1}{K_{NBP}}\right) + 4.35$ N=15, r^2=0.88, s=0.44	NBP	This was probably the first QSAR using chemical reactivity as descriptor to predict fish lethality. No correlation was observed with hydrophobicity
Hermens et al., 1987	Aquatic toxicity (guppy)	NBP	Organophosphorus	$\log\frac{1}{LC_{50}} = 0.23(\pm 0.08)\sum\pi + 0.80(\pm 0.12)\log K_{NBP} + 2.77$ N=9, r^2=0.92, s=0.19	NBP, π (hydrophobicity)	The inclusion of K_{NBP} into the models significantly improved the predictability. The authors recognized the utility of the NBP assay although it could only take into account S_N mechanisms.

European Commission

EUR 24870 EN – Joint Research Centre – Institute for Health and Consumer Protection
Title: The Use of Chemical Reactivity Assays in Toxicity Prediction
Author(s): David Asturiol and Andrew Worth
Luxembourg: Publications Office of the European Union
2011 – 38 pp. – 21 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1831-9424 (online), 1018-5593 (print)
ISBN 978-92-79-20641-2
doi: 10.2788/32962

Abstract

The use of so-called "*in chemico*" methodology - abiotic assays that measure chemical reactivity - is gaining ground as relevant and reliable means of toxicity prediction. In this report we explain the basis of the *in chemico* approach to toxicity prediction and we review the studies that have developed the concept and its practical application since the 1930s, with special attention being paid to studies aimed at the development of Quantitative Structure-Activity Relationship (QSAR) models or read-across approaches. The studies covered in this review are limited to non-enzymatic experiments and to nucleophiles up to 50 amino acids. The main applications identified are related to the assessment of skin sensitisation, aquatic toxicity and hepatotoxicity. Various experimental measures of nucleophile depletion or adduct formation have been proposed as chemical reactivity descriptors, but no single protocol has emerged as the most generally useful. It is concluded that *in chemico* approaches provide a promising means of toxicity prediction within their applicability domains and should be further developed and investigated as alternative methods to animal testing, especially when used in the context of integrated testing strategies based on the use of multiple non-animal methods.

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EUROPEAN COMMISSION

LB-NA-24870-EN-N

Publications Office

ISBN 978-92-79-20641-2